1981

Spectrophotometric determination of thiocyanate enhanced with ethoxyethanol; Spectrophotometric assay of rhodanese.

Avrum Aubrey. Goldstein
University of Windsor

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LA THÈSE A ÉTÉ
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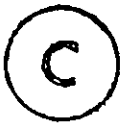
I SPECTROPHOTOMETRIC DETERMINATION OF THIOCYANATE ENHANCED WITH ETHOXYETHANOL

II SPECTROPHOTOMETRIC ASSAY OF RHODANESE

By



Avrum Aubrey Goldstein

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario

1980

752229

ABSTRACT

PART I

SPECTROPHOTOMETRIC DETERMINATION OF THIOCYANATE ENHANCED WITH ETHOXYETHANOL

by

Avrum Aubrey Goldstein

A spectrophotometric method for the determination of thiocyanate using ferric chloride in ethoxyethanol and hydrochloric acid is described. Simplex optimization is used to optimize the volume of acid, the temperature and ratio of reagent to sample. Beer's law is obeyed and the molar absorptivity at 468 nm is 1.954×10^{3} L cm^{-1} mol^{-1}. The method is simple and reproducible.

PART II

SPECTROPHOTOMETRIC ASSAY OF RHODANESE

by

Avrum Aubrey Goldstein

The spectrophotometric determination of thiocyanate is applied to the assay of rhodanese activity in a crude liver homogenate. Various agents are tested to stop the reaction. Trichloroacetic acid is found to be the best reagent for this purpose.

DEDICATION

To my parents, Maurice and Esther.

ACKNOWLEDGEMENTS

I gratefully acknowledge the invaluable assistance I received from my advisors; Dr. R. J. Thibert for introducing me to Clinical Chemistry, Dr. B. Zak for the many articles, references and ideas which he provided, Dr. T. F. Draisey for giving me the run of his hospital and showing me the real world of the medical laboratory, and Dr. K. E. Taylor for his explanations and guidance in the solution of experimental and other problems.

I thank my fellow students for their comments, aide and good humour which saw me through good times and bad.

Many thanks must go to K.J.D. and D.V.D. The former convinced me to finish this work and the latter helped make it a reality.

Special thanks go to Denise who added this work to her busy schedule without complaint.

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PART I

SPECTROPHOTOMETRIC DETERMINATION OF THIOCYANATE ENHANCED WITH ETHOXYETHANOL

CHAPTER I

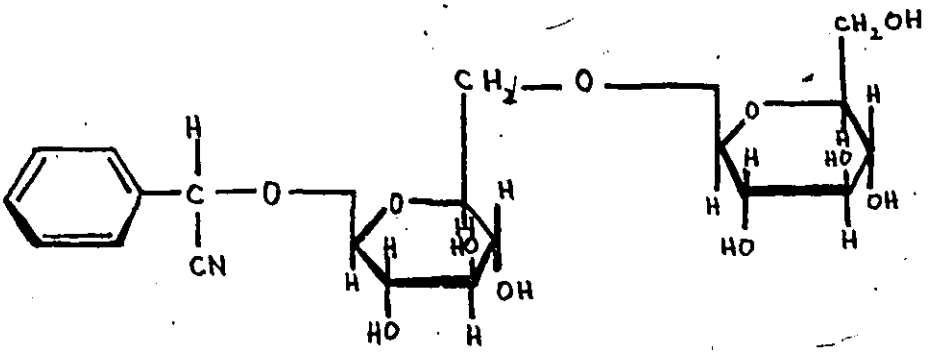
INTRODUCTION

Recent journals have been filled with articles concerning the controversial compound: laetrile (1-34). Laetrile is a cyanogenic glycoside that is an effective anti-cancer drug according to its proponents. Opponents claim laetrile is useless at best and dangerous at worst.

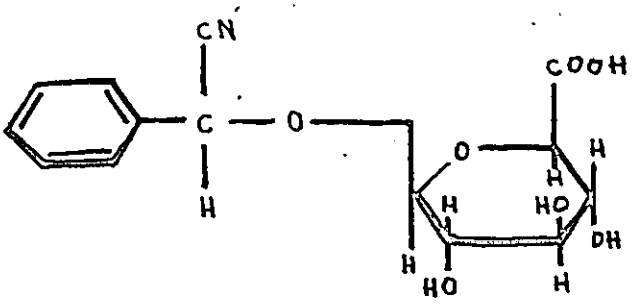
Laetrile, sold as pills or in injectable form, is in fact amygdalin, a related compound containing an additional sugar moiety (Figure I). Amygdalin is a natural product that can be extracted from the kernels of almonds, apples, apricots, cherries, peaches, pears and plums (2). β-Glucosidase cleaves the sugar molecules off amygdalin and the resulting mandelonitrile spontaneously decomposes to release cyanide in the cell. It is this cyanide which accounts for the death of the cancerous tissue according to the champions of laetrile. They claim that normal non-cancerous tissue contains sufficient rhodanese to detoxify cyanide (by converting it to thiocyanate). Cancerous tissue, in this hypothesis, is said to contain little or no rhodanese activity. This makes it susceptible to inhibition of cellular respiration by cyanide.

FIGURE 1

AMYGDALIN AND LAETRILE



D-Amygdalin



Laetrile

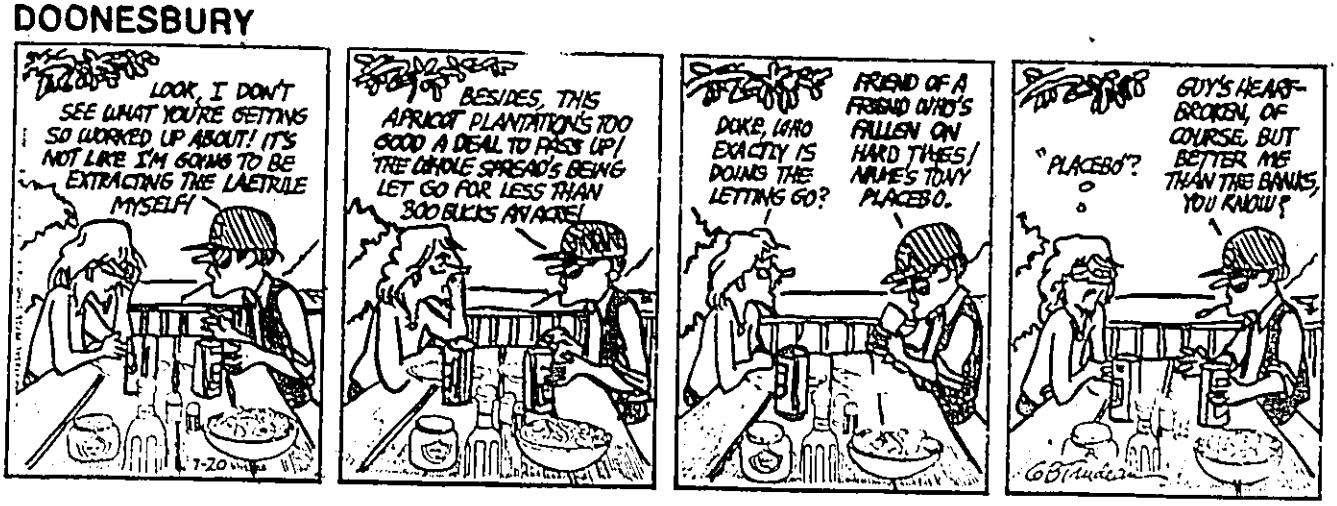
Results of early studies on rhodanese levels in cancerous and non-cancerous tissues were variable (35, 36). A number of tissues had less rhodanese activity when cancerous than when non-cancerous. However, some malignant tissues contained much more rhodanese than corresponding normal tissue. No pattern or explanation was found.

Amygdalin has been tested on mice and dogs with no evidence of remission or cure (5-8, 37, 38). One study found survival times shortened by the administration of amygdalin (8). Experiments on humans have shown similar results (10, 39, 40). Pharmacologic studies have shown that parenterally injected amygdalin passes through the human body unchanged (11). Virtually 100 percent of all the injected dose was recovered in the urine.

The legislatures of many provinces, states and countries are tackling the question of legalizing laetrile. Approximately fifteen states in the United States have legalized the selling of this compound for personal use. Scientists and physicians are being called upon to provide evidence supporting or contraindicating the efficacy of laetrile, which is still popular in the press (Figure 2). Renewed examination of its mechanism of action could prove valuable towards this end.

FIGURE 2

LAETRILE IN THE PRESS



Taken from Detroit Free Press,1979.

In this thesis two series of experiments are detailed concerning part of the mechanism of action discussed previously. The first set of experiments involves the improvement of existing methods of determining thiocyanate. The second outlines a fairly simple and rapid method of assaying the level of rhodanese activity.

CHAPTER II

SPECTROPHOTOMETRIC DETERMINATION OF THIOCYANATE

INTRODUCTION

A number of methods are available for determination of thiocyanate (41-69). Although ion-selective electrodes specific for thiocyanate have been developed (60-63), most laboratories measuring this anion use spectrophotometric methods. Many of these procedures employ iron-based reagents. When the iron reagent is added to a volume of sample the resulting solution is coloured. This colour can be measured and compared to standard solutions.

The available methods suffer from a lack of sensitivity and generally have low molar absorptivity. This leads to rather high limits of detection, not entirely suitable for clinical adaptation. These problems were examined by first looking at the present iron methods, and second by altering them.

EXPERIMENTAL

Materials

Thiocyanate standard solutions (1000 μmol/L):

Prepared by dissolving 97.2 mg reagent grade potassium thiocyanate [Fisher Scientific Co. (FSC)] in one liter of distilled deionized water. Working solutions of lower concentration were prepared by further diluting the stock solution with distilled deionized water.

Ferric nitrate reagent (10g/L): Prepared by dissolving reagent grade ferric nitrate (FSC) in 1.5 N nitric acid.

Ferric chloride solution (4.1564 g/L), aqueous: Prepared by dissolving reagent grade ferric chloride (FSC) in 0.1 N hydrochloric acid.

Ferric chloride solution (4.1564 g/L), methoxyethanol: Prepared by dissolving reagent grade ferric chloride (FSC) in 0.834 mL hydrochloric acid and diluting to 100 mL with spectral grade methoxyethanol (FSC).

Ferric chloride solution (4.1564 g/L), 1-butanol: Prepared by dissolving reagent grade ferric chloride (FSC) in 0.834 mL hydrochloric acid and diluting to 100 mL with reagent grade 1-butanol (FSC).

Ferric chloride solution (4.1564 g/L), 1-pentanol: Prepared by dissolving reagent grade ferric chloride (FSC) in 0.834 mL hydrochloric acid and diluting to 100 mL with reagent grade 1-pentanol (FSC).

Ferric chloride solution (4.1564 g/L), butoxyethanol: Prepared by dissolving reagent grade ferric chloride (FSC) in 0.834 mL hydrochloric acid and diluting to 100 mL with reagent grade butoxyethanol (FSC).

Ferric chloride solution (4.1564 g/L), ethoxyethanol: Prepared by dissolving reagent grade ferric chloride (FSC) in 0.834 mL hydrochloric acid and diluting to 100 mL with reagent grade ethoxyethanol (FSC).

Ferric chloride solutions (0.6926 g/L), ethoxyethanol: Prepared by dissolving reagent grade ferric chloride hexahydrate (FSC) in various volumes of hydrochloric acid and diluting to 100 mL with reagent grade ethoxyethanol (FSC).

Apparatus

Micropipettors: Oxford, available through Canadian Laboratory Supplies, Ltd., Toronto, Ontario.

Vortex Mixer: Vortex Jr. Mixer, Scientific Industries, Inc., Springfield, Mass., 01103, U.S.A.

Spectrophotometer: Beckman Acta MVI, Beckman Instruments, Inc., Analytical Instruments Sales & Service Division, Toronto, Ontario.

Methods

An aliquot of thiocyanate solution was transferred to

a small test-tube. To this was added an aliquot of the iron reagent being examined. The resulting solution was mixed using a vortex mixer.

The solution was then transferred to one-centimeter quartz cuvettes which were placed in the spectrophotometer. A wavelength scan from 400 to 700 nm was made to determine the wavelength of maximum absorbance. The temperature of the sample chamber, and therefore the samples, was varied. The maximum absorbance at optimum wavelength and temperature was noted and monitored for a length of time, up to 45 minutes.

After discovering a large improvement when using ethoxyethanol as the solvent, Simplex optimization was employed to find the optimum conditions for this assay. The variables modified using this method included: the volume of hydrochloric acid; the temperature of the sample during measurement of absorbance; and the ratio of reagent to sample. The computer program and other details of Simplex optimization will be discussed in CHAPTER III, PART I.

RESULTS AND DISCUSSION

The wavelength scan of the ferric nitrate reagent

plus the sample showed a maximum at 460 nm. A 2:1 ratio of reagent to sample volume was used. The sample temperature was maintained at 25°C.

A variety of sample concentrations were examined (Table I) and a standard curve calculated. This curve was fairly linear to 500 µmol/L thiocyanate.

The effect of reagent to sample ratio was examined (Table II). Ratios between 1:1 and 5:1 were tested. The optimum for this system occurred at 1.5 volumes reagent to every one volume of sample.

In order to examine this system to find the number of iron atoms associated with the thiocyanate ion, an experiment to determine the formula of the complex was carried out. The results of this experiment were inconclusive (Table III). As more and more iron was added to the system the absorbance of the sample continued to increase. Bowler (49) investigated this problem and found that a very large excess of ferric nitrate was required to produce maximum colour. Partial hydrolysis at this concentration resulted in increased blank absorbance.

A major problem with the ferric nitrate method was the fast fading of the colour. Within ten minutes the absorbance decreases between 0.5 and 1.5 absorbance units,

TABLE I

ABSORBANCES OF THIOCYANATE SAMPLES
USING FERRIC NITRATE REAGENT

Concentration of thiocyanate μmol/L	Absorbance
20	0.017
50	0.047
100	0.095
250	0.225
500	0.489

Assay Protocol: 2 mL of ferric nitrate reagent were added to 1 mL of a standard thiocyanate solution, mixed and the absorbance read at 460 nm while the cuvettes were maintained at 25°C. Spectrophotometer was zeroed according to the Manual using appropriate blank solutions. This procedure was followed for all measurements made for this research. Thiocyanate concentrations are those of stock solutions and not final concentrations. This is true of all concentrations quoted. The absorbance readings above are the average of triplicate analyses.

TABLE II

THE EFFECT OF REAGENT/SAMPLE RATIO

Volume of ferric nitrate reagent (mL)	Volume of thiocyanate (mL)	Absorbance
1.0	1.0	0.494
1.5	1.0	0.561
2.0	1.0	0.489
2.5	1.0	0.426
3.0	1.0	0.383
3.5	1.0	0.334
4.0	1.0	0.309
4.5	1.0	0.276
5.0	1.0	0.255

Assay Protocol: Conditions were those described in Table I except that the reagent volume was varied as shown. The thiocyanate concentration was kept constant at 500 µmol/L. The absorbance readings above are the average of triplicate analyses.

TABLE III

FORMULA OF A COMPLEX:
MOLAR-RATIO METHOD

Volume of SCN^- (mL)	Volume of 1.5 N HNO_3 (mL)	Volume of ferric nitrate (mL)	Molar-Ratio [FNR]/[SCN^-]	Absorbance
3.0	0	17	228.9	0.505
3.0	1	16	215.5	0.495
3.0	2	15	202.0	0.487
3.0	3	14	188.5	0.472
3.0	4	13	175.1	0.453
3.0	5	12	161.6	0.493
3.0	6	11	148.1	0.425
3.0	7	10	134.7	0.407
3.0	8	9	121.2	0.391
3.0	9	8	107.7	0.367
3.0	10	7	94.3	0.343
3.0	11	6	80.8	0.315
3.0	12	5	67.3	0.282
3.0	13	4	53.9	0.246
3.0	14	3	40.4	0.203
3.0	15	2	26.9	0.155
3.0	16	1	13.5	0.084

FNR - ferric nitrate reagent (see Materials)

Assay Protocol: Standard thiocyanate solution (1000 μmol/L) was mixed with the volumes of 1.5 N HNO_3 and FNR solutions. The absorbance was measured at 460 nm and a constant temperature of 25°C. Separate blanks were used for each solution. The absorbance readings above are the average of triplicate analyses.

depending on the concentration of thiocyanate in the sample.

Ferric chloride in water and in 2-methoxyethanol were examined next. Wavelength scans found maximum absorbances to occur at 460 and 470 nm for aqueous and methoxyethanol based solutions, respectively. Other maxima were observed at 370 and 380 nm and were thought to be due to the iron ion itself.

In 1937, Winsor published a paper (67) detailing a study of ferric thiocyanate colour using 2-methoxyethanol as the solvent. His interest was the determination of iron using thiocyanate. He reported an 85 per cent increase in colour intensity when compared to water based systems. He claimed that the low dielectric constant of this organic solvent (at that time reported as being 12.0) prevents or helps to prevent dissociation of the iron-thiocyanate complex. This solvent is also considered ideal because of its low volatility, its lack of colour when combined with thiocyanate (with no iron present) and its infinite miscibility with water.

Comparison of the absorbances of the ferric chloride solutions with 250 and 500 μmol/L thiocyanate samples showed improvements over the ferric nitrate reagent. The methoxyethanol based reagent being better than the

aqueous reagent. However, this improvement was nowhere near the 85 per cent claimed by Winsor (Table IV). Some fading of the colour was observed with both reagents, though the degree of fading did not approach that of the ferric nitrate reagent system.

At this point the paper by Kita et al.(57) using a methanol-water solution purporting to give increased absorbance to the iron-thiocyanate complex was examined at length. The claim was made that the presence of the methanol (50%) added stability and 'tightened' the complex. This lead to increased intensity of the resulting colour. It was proposed that a number of complexes exist in solution and that the organic solvent 'pushed' the equilibria in the direction of the most coloured complex.

From this conclusion the decision was made to examine the effect of a number of alcohols on the ferric chloride reagents. The criteria used to select these alcohols was their dielectric constant and its relation to that of the water-methanol system (Table V). 1-Butanol, 1-pentanol, 2-butoxyethanol, 2-methoxyethanol and 2-ethoxyethanol were found to have dielectric constants relatively close to that of the mixture used by Kita et al.(57).

TABLE IV

COMPARISON OF VARIOUS ORGANIC SOLVENT SYSTEMS

Solvent	Wavelength of max. absorbance	Ethanol added	Concentration of SCN^- µmol/L	Absorbance	Percentage change
Water	460 nm	----	250	0.264	---
			500	0.528	---
1-Butanol	465 nm	1 mL	250	0.242	- 8.3
			500	0.462	-12.5
1-Pentanol	455 nm	2 mL	250	0.219	-17.0
			500	0.402	-23.9
Butoxy-ethanol	468 nm	----	250	0.101	-61.7
			500	0.360	-31.8
Methoxy-ethanol	470 nm	----	250	0.296	12.1
			500	0.602	14.0
Ethoxy-ethanol [a]	468 nm	----	250	0.326	23.5
			500	0.622	17.8

a. Initial experiment

Refer to Methods and Results and Discussion for details. The absorbance readings above are the average of triplicate analyses.

TABLE V

DIELECTRIC CONSTANTS OF
SELECTED ORGANIC SOLVENTS

Solvent	Dielectric constant 25°C
Water	78.54
Methanol	32.63
1-Butanol	17.51
2-Butanol	16.56
1-Pentanol	13.90
2-Pentanol	13.82
3-Pentanol	13.02
2-Methoxyethanol	16.93
2-Ethoxyethanol	29.60

Data from: CRC Handbook of Chemistry and Physics, ed. R.C. Weast and M.J. Astle, CRC Press, Inc., Boca Raton, Florida, 33431, 1978.

International Critical Tables of Numerical Data, Physics, Chemistry and Technology, Volume VI, National Research Council, McGraw-Hill Book Company, Inc., New York and London, 1929.

Sammour et al. (64) examined the effect of various acids and anions on the formation of the iron-thiocyanate colour. They reported that 0.1 N hydrochloric acid produced the highest absorbance of the measurements made with this acid. Increased acid concentration lowers absorbance. Therefore all trials of various systems were initiated using solvents 0.1 N in hydrochloric acid.

The experiments performed using 1-butanol as the solvent had to be modified because butanol and water are not miscible to a sufficient degree. In order to overcome this, 1.0 mL of ethanol was added to each tube prior to mixing. This produced an homogenous solution. However, trials with both 250 and 500 µmol/L thiocyanate levels produced lower absorbances than trials with aqueous reagents (Table IV).

2-Butoxyethanol was tested as a solvent for the iron-thiocyanate reaction and found to have maximum absorbance at 468 nm. Absorbance values for both thiocyanate levels, 250 and 500 µmol/L, were much lower than those recorded for the aqueous reagent. Further problems include fading of the iron-thiocyanate colour and the toxicity of the solvent itself.

2-Ethoxyethanol was the last solvent examined. It

has a higher dielectric constant than 2-methoxyethanol, 29.60 versus 16.93, respectively. Based on Winsor's suppositions about the effect of this property, we would not expect 2-ethoxyethanol to be a better solvent than 2-methoxyethanol. However, Winsor is wrong.

Maximum absorbance occurred at 468 nm. An increase in absorbance over both aqueous and methoxyethanol systems is seen using ethoxyethanol as the solvent (Table IV). Initial trials showed increases averaging 20.6 per cent compared to aqueous reagents. Using Simplex optimization (described in CHAPTER III) to vary the volume of acid, the temperature of the system and the ratio of reagent to sample, the procedure was improved. The optimum absorbance occurred using 2.0 mL hydrochloric acid per 100 mL reagent, a reagent to sample ratio of 0.20 to 1.0 and a temperature of 19.5°C. These conditions produced an increase in absorbance of 99.8 per cent.

In order to objectively examine this increase, another series of experiments were run. All samples were run with the appropriate solvent added in order that all trials have reagent to sample ratios of 1 to 1. Table VI allows for comparison of the molar absorptivities calculated from

TABLE VI

COMPARISON OF MOLAR ABSORPTIVITIES OF VARIOUS SOLVENT SYSTEMS

Solvent	Reagent/Sample ratio	Solvent added	Concentration of SCN^- µmol/L	Absorbance	Molar absorptivity[b] ϵ (L/cm-mol)	Change in ϵ%
Water (FNR)	1:1	-----	500	0.489	9.78×10^2	----
Ethoxy-ethanol	1:1	-----	500	0.808	1.616×10^3	65.2%
Ethoxy-ethanol	0.25:1	0.75 mL[a]	500	0.560	1.12×10^3	14.5%
Ethoxy-ethanol	0.20:1	-----	500	0.977	1.954×10^3	98.8%

a. Volume of solvent added in order to maintain 1 to 1 reagent to sample ratio.

b. Molar absorptivity measured at 460 nm for FNR and 468 nm for all others.

The absorbances above are the average of triplicate analyses.

these results. A 65.2 per cent increase in molar absorptivity was recorded for one trial using ethoxyethanol as compared to aqueous solvents. Altering the optimum conditions (in the previous paragraph) results in an abrupt decrease in absorbance; although there was a 14.5 per cent increase in molar absorptivity.

SUMMARY AND CONCLUSIONS

A spectrophotometric method employing ethoxyethanol as the solvent for the iron-based determination of thiocyanate has been presented. It has been compared to other solvents and found to offer a large increase in molar absorptivity.

Using pure samples (no protein) the absorbance recorded for 500 μmol/L thiocyanate solutions increased from 0.489 using FNR with water as the solvent to 0.977 employing ferric chloride in ethoxyethanol. This represents an increase of 99.8 per cent.

However this solvent, 2-ethoxyethanol, is not without drawbacks. It is a hazardous compound, damaging the central nervous system by inhalation or by absorption through the skin. It should be used in a well ventilated area or under a fume hood (70, 71).

CHAPTER III

SIMPLEX OPTIMIZATION OF THIOCYANATE DETERMINATION

INTRODUCTION

The optimization of an assay procedure has been much simplified by the introduction of a new modification process: Simplex optimization. This process eliminates numerous experiments where one variable is adjusted slightly while the others are held constant. Using this new method all variables can be adjusted simultaneously and the optimum conditions found in a much shorter time period (72-74).

Simplex optimization was used to maximize the absorbance readings for a given concentration of thiocyanate. Therefore absorbance is the response being monitored.

> "Running experiments at enough combinations of levels gives responses whose magnitudes can be used to graphically depict a response surface. This surface resembles a mountain, with the maximum response lying at the summit.....
>
> To maximize a response, the problem is to locate the coordinates of the summit of the response surface, these coordinates being the optimum magnitude of the factors affecting the response. To accomplish this goal, the factors governing the conditions in a set of experimental runs are given different levels according to a definite plan. Comparing the responses resulting

> from a correctly planned set allows one to predict the direction in factor space for experimental conditions capable of even higher response." (72)

The clearest example is a two-factor simplex in which the third factor is being optimized. Three experiments are run and their coordinates plotted on a graph similar to a topographical map, on which the highest hill represents the maximum response. These three experiments form a triangle on the map. The worst response is eliminated by reflecting it through the opposite side of the triangle (its hyperface) as its mirror image. The coordinates of this point are then used to run a fourth experiment. After this experiment is run the responses are again compared and the poorest eliminated. A new simplex is formed and a new experiment run using the newly calculated coordinates of the fifth vertex as the new conditions. In this way the simplex is employed to approach the highest hill or the maximum response possible.

This example used only two factors. However any number of factors may be examined, although this increases the complexity of the calculations and the number of experiments necessary. The number of experiments required is 2^k where k is the number of factors being investigated

(72). Therefore it is preferable to concentrate on a limited set of important factors.

Other significant considerations include the location of the first simplex and its size. Generally, a large simplex size is initially valuable as the maximum response may be approached quickly. The drawback of this is that the simplex may overshoot the maximum. Hand in hand with step size is location of the initial simplex. The first experiments can be based on present assay conditions or those suggested by recent work.

One final consideration is the setting of limits or constraints on the simplex. These limits represent undesirable conditions. For example, a temperature limit may be set in order to avoid sample denaturation. If the simplex crosses the limit it must be redirected. Deming and Morgan (73) report a series of rules for the use of simplex optimization:

> "Rule 1: A move is made after each observation of response.
> Rule 2: A move is made into that adjacent simplex which is obtained by discarding the point of the current simplex corresponding to the least desirable response and replacing it with its mirror image across the (hyper) face of the remaining points.
> Rule 3: If the reflected point has the lease desirable response in the new simplex, do

not reapply rule 2, but instead reject the second lowest response in the new simplex and continue.

Rule 4: If a vertex has been retained in k + 1 simplexes, (where k is the number of dimensions of the simplex) before applying rule 2 reobserve the response at the persistant vertex.

Rule 5: If a new vertex lies outside the boundaries of the independent variables, do not make an experimental observation but instead assign to it a very undesirable response." (73)

It is possible to arrive at a maximum which is not the ultimate maximum. (Imagine a series of foothills surrounding a mountain.) By using a new simplex located away from previous simplexes and the current maximum, this possibility may be checked. If the new simplex returns towards the previous maximum, it probably represents the optimum conditions.

EXPERIMENTAL

The rules mentioned previously were used in the simplex optimization of the ethoxyethanol based thiocyanate system discussed in CHAPTER II. Three variables were examined: the volume of concentrated hydrochloric acid in the ferric chloride reagent, the temperature of the samples and the reagent volume to sample volume ratio. These variables are noted as pH, TEMP and RATIO, respectively, in the computer program.

The program was written to aid in the calculation of the new simplex. The program is based on calculations suggested by Long (72). The equation is in Figure 3. Table VII contains the computer program, as used.

RESULTS AND DISCUSSIONS

The majority of the results are found in Tables VIII and IX. These two tables show very different results obtained using the same simplex optimization procedure. Table VIII shows a gradual improvement of the response. While in Table IX the simplex seems to have 'lost its way', wandering about, violating the arbitrary temperature barrier.

It is possible that the location and step size of the original simplex in Table IX were poorly chosen. This can lead to the wandering observed. Eventually this seemingly random motion should have lead to a maximum response, according to Long (72). By applying the rules and doing enough experiments simplex optimization is an effective method for improving chemical analysis methods, results in Table IX not withstanding.

The optimum conditions (Table VIII) occur when 1.00 mL of concentrated hydrochloric acid is added to the ferric

FIGURE 3

CALCULATION OF A NEW VERTEX

$$\frac{2}{k} \quad (S) \quad - \quad DV \quad = \quad NV$$

k = number of vertices

S = sum of retained coordinates

DV = coordinates of discarded vertex

NV = coordinates of new vertex

Figure proceeds through points in numerical order.

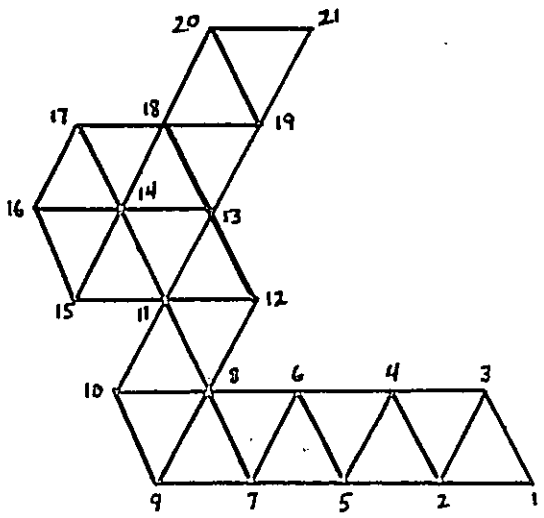


TABLE VII

COMPUTER PROGRAM FOR SIMPLEX OPTIMIZATION

```
 1.           INTEGER A, B, T, V, X
 2.           REAL Z (10, 3), W (1, 3)
 3.           READ 1, ([Z (A, B), A=1, 10], B=1, 3)
 4.     1     FORMAT (10 F5.3/10 F5.3/10 F5.3)
 5.           READ 2, ([W (T, V), T-1, 1], V=1, 3)
 6.     2     FORMAT (3 F5.3)
 7.           T = V = 1
 8.           X = 0
 9.           DO 3 B = 1, 3
10.           SUM = 0
11.           DO 4 A =1, 10
12.           SUM = SUM + Z (A, B)
13.           IF (Z [A, B] .EQ.0) GO TO 5
14.     4     CONTINUE
15.     5     Z (A, B) = SUM
16.           Z (A + 1, B) = SUM * 2/(A-2)
17.           Z (A + 2, B) = Z (A + 1, B) -  W (T, V)
18.           V = V + 1
19.           X = A + 2
20.     3     CONTINUE
21.           PRINT 6
22.     6     FORMAT (' ', 10X, 'PH', 5X, 'TEMP', 4X, 'RATIO')
23.           PRINT 7, ([Z (A, B),B = 1, 3], A = 1, X)
24.     7     FORMAT ('0', 8X, 3(F6.2, 2X)//(9X, 3(F6.2, 2X)//)
25.           STOP
26.           END
```

TABLE VIII

SIMPLEX OPTIMIZATION OF THIOCYANATE DETERMINATION

Vertex number	Vertices retained	Variables			Response absorbance [h]	Notes
		Volume HCl	Temp	Ratio		
1.		0.83	23.30	1.00	0.810	a
2.		1.50	25.00	2.00	0.544	
3.		0.40	27.00	3.00	0.545	
4.		0.32	25.20	2.00	0.682	
5.	1,3,4	1.12	23.80	1.00	0.737	
6.	1,2,4	0.02	25.25	2.00	0.577	b
7.	1,5,4	1.19	23.00	0.67	0.745	
8.	1,5,7	0.97	22.93	0.78	0.578	
9.	1,7,8	1.02	23.22	0.85	0.767	c
10.		0.83	23.30	1.00	0.748	d
11.	7,9,10	0.84	23.35	1.01	0.794	
12.	10,9,7	1.03	12.19	0.87	0.770	
13.	12,11,9	1.03	23.25	0.86	0.779	
14.	11,13,12	0.94	23.33	0.94	0.777	
15.	11,13,14	0.89	23.32	0.97	0.821	e
16.	11,13,15	0.91	23.29	0.95	0.819	
17.		1.03	23.25	0.86	0.826	f
18.	17,15,16	1.00	23.26	0.89	0.830	g

TABLE VIII continued

a. Temperature recorded and calculated to two decimal places but used in practice to only one. Adjusted to closest tenth.

b. Vertices 2. & 3. are so close both were used in calculation of vertices 6. & 5., respectively.

c. Newest vertex in the previous simplex was the worst. Rule 3 applied.

d. 10. is a repeat of 1. Absorbance depends on accurate $FeCl_3$ weighing. May need new standard iron source.

e. Made with $FeCl_3.6H_20$ as are all following solutions.

f. 17. is repeat of 13. with $FeCl_3.6H_20$.

g. In order to find out if this is the true optimum, start from new area.

h. Absorbances measured at 468 nm. Absorbances are the average of triplicate analyses.

TABLE IX

SIMPLEX OPTIMIZATION OF THIOCYANATE DETERMINATION

Vertex number	Vertices retained	Variables			Response absorbance[e]	Notes
		Volume HCl	Temp	Ratio		
19.		5.00	20.00	1.00	0.516	
20.		3.00	30.00	1.25	0.553	
21.		7.50	25.00	0.80	0.458	
22.		10.00	23.30	3.00	0.154	
23.	20,19,21	7.00	42.23	1.03	0.445	
24.	20,19,23	1.52	25.42	0.73	0.788	a
25.	24,20,19	4.01	36.20	1.64	0.450	
26.	24,20,25	4.02	54.41	2.08	- 0.100	a, b
27.	24,25,26	5.36	21.14	2.55	0.278	
28.	24,25,27	5.92	31.15	2.58	0.120	
29.	24,25,28	5.85	54.80	2.45	- 0.100	a, b
30.	24,25,29	5.61	20.83	2.35	0.267	
31.	24,25,30	6.47	30.30	2.33	0.238	
32.		6.47	30.30	1.00	0.463	c
33.	24,32,25	5.84	51.18	1.47	- 0.100	b
34.	24,32,33	7.88	17.62	1.58	0.374	
35.	24,32,34	8.63	23.59	1.71	0.321	a
36.	24,32,35	8.45	47.00	1.76	- 0.100	b
37.	24,32,36	8.08	17.95	1.75	- 0.350	
38.	24,32,37	7.89	37.44	1.73	0.280	
39.	24,32,38	7.89	57.12	1.72	- 0.100	a, b
40.	24,32,39	7.95	52.10	1.72	- 0.100	b
41.	24,32,40	7.99	29.76	1.72	0.342	
42.	24,32,41	8.00	36.56	1.73	0.318	d

TABLE IX continued

a. Newest vertex in previous simplex was the worst. Rule 3 applied.

b. Boundary violation, temperature greater than 40°C. Rule 5 applied.

c. Redirect simplex.

d. Simplex is wandering, not approaching optimum.

e. The absorbance readings are the average of triplicate analyses.

chloride reagent, the samples are analyzed at 23.3°C, and the reagent to sample ratio is 0.89 volumes of reagent to 1.00 volume of sample. Further experiments improved the assay procedure and resulted in an absorbance of 0.977 at a temperature of 19.5°C, a reagent to sample ratio of 0.20 mL to 1.0 mL, and adding 2.0 mL of hydrochloric acid per 100 mL of reagent. Tables VIII and IX are included as examples of simplex optimization. The simplex resulting in the final optimum conditions is not included in the data.

PART II

SPECTROPHOTOMETRIC ASSAY OF RHODANESE

CHAPTER I

INTRODUCTION

The first report of rhodanese activity was published in 1933 (75). Rhodanese (Thiosulphate: Cyanide Sulphurtransferase) converts cyanide to thiocyanate in an almost completely irreversible reaction. Since that first report rhodanese has been widely studied (75-95). Many important characteristics and properties of this enzyme have been discovered.

Rhodanese is a mitochondrial enzyme found in high concentration in mammalian liver and kidney tissue. Smaller amounts are found in erythrocytes, brain tissue and bacteria such as E. coli.

The enzyme employs thiosulphate, thiosulphonates, persulphides, and polysulphides as sulphur donors. The sulphur acceptors include cyanide, sulphite, organic sulphinates, thiols and dithiols, borohydride and dithionite (86). The following reaction shows how rhodanese (Rh) removes sulphur from the donor molecule and adds it to the acceptor.

$$Rh + S_2O_3^{=} \rightleftharpoons Rh\text{-}S + SO_3^{=}$$
$$Rh\text{-}S + CN^{-} \rightleftharpoons Rh + SCN^{-} \qquad (85)$$

Originally reported as a dimer of molecular weight 36,500, X-ray crystallography has proven otherwise. A recent paper (89) reports rhodanese to be a single polypeptide chain of 293 residues. The molecular weight based on this finding was 32,900. The molecular shape was described as a ellipsoid.

Rhodanese's primary, and for many years only function, was thought to be the detoxification of cyanide by its conversion to thiocyanate. It appears today that this may not be its most important function, (even though administration of thiosulphate and rhodanese has proven to be a successful treatment for cyanide poisoning). Westley (75) has reported a number of other roles for this enzyme. Disposal of sulphide (a very neurotoxic compound) formed from cysteine by the enzyme cystathionase was mentioned. Reduction of thiosulphate and the transfer of reduced sulphur is another known function. A related role employs rhodanese to produce reduced sulphur for biosynthesis as in the conversion of serine to cysteine.

Rhodanese is important to this work because of its

originally suggested function. Laetrile releases cyanide into the cells where it can be detoxified by the action of rhodanese. In the absence of this enzyme, cellular respiration ceases and cell death soon follows. Therefore the levels of this enzyme in normal and cancerous tissue are important for the proposed mechanism of action of this unproven 'cure'. By examining these levels, support or contradiction of this hypothesis can be realized.

Nitroprusside is a compound related to laetrile. It contains five cyanide molecules compared to laetrile's single cyanide. Sodium nitroprusside has been used extensively to treat hypertension, acute myocardial infarction, congestive heart failure, mitral regurgitation, aortic regurgitation and to produce hypotension during anesthesia (42, 43). Although its exact mechanism of action is not known, it has been proposed that the release of cyanide is quickly followed by its conversion to thiocyanate, a known hypotensive agent. This conversion is catalyzed by rhodanese. Low levels of this enzyme could lead to death by cyanide poisoning.

The following is a suggested method of testing tissue samples for rhodanese activity.

CHAPTER II

EXPERIMENTAL

Materials

Beef liver: Obtained from Windsor Packing Company, Limited, Tecumseh Rd., Windsor, Ontario.

Glycine Acetate buffer (0.2M): Prepared by combining 0.2M solutions of glycine (15.01 g/1L) and sodium acetate (27.2 g $C_2H_3O_2Na.3H_2O$/1L), both reagent grade (Fisher Scientific Company), and adjusting the pH to 5.0 with base or acid.

Tris-(hydroxymethyl)-aminomethane (Tris) buffer: Prepared by dissolving 24.2 g reagent grade Tris (FSC) in 1L distilled deionized water. A pH of 7.9 was achieved by combining 50 mL of 0.2M Tris with 29.2 mL of 0.2M HCl and diluting to 200 mL.

Potassium thiosulphate solutions: Prepared by dissolving reagent grade $K_2S_2O_3.1/3\ H_2O$ (FSC) in distilled deionized water.

Potassium cyanide solutions: Prepared by dissolving reagent grade potassium cyanide (FSC) in distilled deionized water.

Iron Reagent: Prepared by dissolving 0.69261 g reagent

grade ferric chloride hexahydrate (FSC) in 2.0 mL concentrated hydrochloric acid and diluting to 100 mL with reagent grade ethoxyethanol (FSC).

Formaldehyde, 15%: Prepared by diluting 40.5 mL of 38% formaldehyde solution (FSC) to 100 mL with distilled deionized water.

Zinc sulphate - barium hydroxide precipitant: Zinc sulphate solution prepared by dissolving $ZnSO_4.7H_2O$ in distilled deionized water. Barium hydroxide solution prepared by dissolving $Ba(OH)_2.8H_2O$ in distilled deionized water. The two solutions were combined in sufficient proportion to produce a neutral solution.

Zinc sulphate - sodium hydroxide precipitant: Reagent grade zinc sulphate and sodium hydroxide (FSC) are dissolved in distilled deionized water. They were then combined to form a neutral solution. Reagent grade hydrochloric acid may be added to dissolve amphoteric zinc hydroxide.

Trichloroacetic acid (TCA) precipitant: Prepared by dissolving 5, 10, 20 or 30 g reagent grade TCA (FSC) in 100 mL distilled deionized water.

Chloral hydrate solution: Prepared by dissolving

reagent grade chloral hydrate (FSC) in distilled deionized water.

Sodium hydroxide, saturated: Prepared by dissolving reagent grade sodium hydroxide (FSC) in a volume of distilled deionized water just sufficient to cause it to go into solution.

Sulphanilic acid (0.5%): Prepared by dissolving 0.5 g reagent grade sulphanilic acid (FSC) in distilled deionized water and adjusting the pH to 8.3 by the addition of 0.1 N NaOH.

Pyridine (0.25%): Prepared by diluting 0.25 mL reagent grade pyridine (FSC) to 100 mL with distilled deionized water.

Sodium hydroxide - boric acid buffer (pH 8): Prepared by adding 50 mL of 0.1 M boric acid (FSC) to 3.97 mL of 0.1 N NaOH (FSC) and diluting to 100 mL.

Cyanide indicator: Prepared by mixing 5 mL of each of the sulphanilic acid and the pyridine solutions above and diluting to 500 mL by addition of the sodium hydroxide - boric acid buffer. Chlorine gas is then bubbled through this solution for 15 minutes.

Sodium hydroxide (3%): Prepared by dissolving 3 g

reagent grade sodium hydroxide (FSC) in 100 mL distilled deionized water.

Standard protein solution (7g/100 mL): Prepared by dissolving 0.7 g bovine serum albumin (Sigma Chemical Company) in 10 mL distilled deionized water.

Biuret reagent: Available from Fisher Scientific Company.

Ammonium hydroxide: Available from Fisher Scientific Company.

Apparatus

Waring blender: Available from Fisher Scientific Company Ltd., 8555 Devonshire Rd., Montreal, Quebec, H4P 2L3.

Sorval Centrifuge: Superspeed RC2-B, automatic centrifuge, Ivan Sorvall Inc., Newtown, Connecticut, 06470, U.S.A.

Micropipettors: Oxford, available through Canadian Laboratory Supplies Ltd., Toronto, Ontario.

Spectrophotometer: Beckman Acta MVI, Beckman Instruments, Inc., Analytical Instruments Sales & Service Division, Toronto, Ontario.

Heating Mantle: Glas-Col Heating Mantle available

from Glas-Col Apparatus Company, Terre Haute, Indiana, U.S.A.

Methods

The liver homogenate was prepared by placing 500 g of beef liver in the Waring blender and adding 200 mL of either the glycine acetate or Tris buffers. The tissue was homogenized for 2 to 5 minutes. The resulting suspension was centrifuged for 30 minutes at 14,000 x g and the precipitate discarded. The pH of the supernatant was adjusted to 7.9 with ammonium hydroxide.

An aliquot of the tissue homogenate, usually 0.1, 0.5 or 1.0 mL, is then added to a small test tube containing the substrates CN^- and SCN^- (1 mL of each, final concentrations were 0.05 M and 0.01 M, respectively). After mixing, the reaction vessel is placed in a water bath at 25°C, this being the optimum incubation temperature (93). After one minute (a variety of times were tested) a 1.0-mL aliquot is removed into a second test tube containing one of the following solutions: 15% formaldehyde, sodium hydroxide - zinc sulphate, barium hydroxide - zinc sulphate, trichloroacetic acid (TCA) or choral hydrate.

The protein precipitate was removed by centrifugation at 15,000 rpm (39,000 x g) and the supernatant poured off

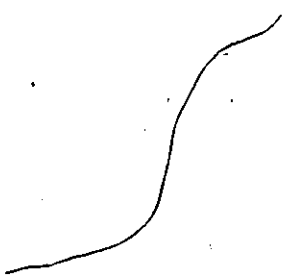
into a third tube. A 1.0-mL aliquot of this solution was then tested for thiocyanate by addition of 0.25 mL of the iron reagent. The absorbance was measured at 468 nm.

A standard curve was prepared by spiking the liver homogenate with a small volume of highly concentrated thiocyanate solution. (Tables X and XI in CHAPTER III compare spiked and unspiked thiocyanate solutions.) The solutions were treated to remove the protein (following the addition of the thiocyanate) with one of the four precipitants mentioned previously. The precipitate was removed by centrifugation at 15,000 rpm.

After many trials prior centrifugation of the liver homogenate for 30 minutes at 15,000 rpm (39,000 x g) was found to be necessary. This prior centrifugation (prior to analysis) removed the red to red-yellow turbidity which remained after the precipitants had been added. The enzyme rhodanese remains in solution while the interfering substances are precipitated out.

The tissue homogenate was tested to determine the amount of protein present using the Biuret reaction (96). The reagent blank was 5 mL of 3% sodium hydroxide. The protein standard was 0.1 mL of the protein solution (7 g/100 mL) added to 4.9 mL of 3% sodium hydroxide. A 0.1-mL

aliquot of the homogenate was combined with 4.9 mL of 3% sodium hydroxide. Biuret reagent (1 mL) was immediately added to each tube and mixed. After incubating for 15 minutes at room temperature, the absorbance of these solutions were measured at 545 nm. From these results the amount of protein, in grams of protein per mL, was calculated.



CHAPTER III

RESULTS AND DISCUSSION

In Table X results for pure thiocyanate standards are shown. The solutions were treated with an equal volume of TCA. The absorbances recorded are slightly higher than those in Table XI. This may be due to some entrapment of the anion in the protein precipitate. Relatively higher blank values may also be the cause of this result.

The standard curve for thiocyanate-spiked liver homogenate is shown in Table XI. All samples were treated with an equal volume of TCA to precipitate the protein. The addition of the ethoxyethanol-based iron reagent produced an intense red-orange colour characteristic of iron-thiocyanate complexes. The curve is linear up to and including 1000 μmol/L.

TCA concentrations of 5, 10, 20 and 30 g/100 mL were examined. The most effective concentration was 20 g/100 mL. At this level all protein was removed from solution and a minimum volume of TCA could be employed. No improvements were noted with higher concentrations of TCA.

Prior to using TCA other precipitants were tried. None of these agents proved suitable. In order to

TABLE X

STANDARD CURVE FOR PURE THIOCYANATE STANDARDS[a]

Tube number	Volume of thiocyanate standard (mL)	Volume of TCA (mL)	µmol/L [KSCN]	Absorbance[c]
1	1.0	1.0	20	0.015
2	1.0	1.0	50	0.035
3	1.0	1.0	100	0.059
4	1.0	1.0	250	0.152
5	1.0	1.0	400	0.240
6	1.0	1.0	500	0.300
7	1.0	1.0	1000	0.605
8	0.0[b]	2.0	--	0.000

a. Assay Protocol: one mL of the centrifuged thiocyanate standard - TCA mixture was transferred to a clean test tube, 0.25 mL of the iron reagent was added and mixed. An aliquot of this solution was then examined spectrophotometrically. TCA concentration - 20 g/100 mL.

b. 2.0 mL distilled deionized water instead of thiocyanate solution.

c. The absorbances are the average of triplicate analyses.

TABLE XI

THIOCYANATE SPIKED LIVER HOMOGENATE

STANDARD CURVE[a]

Tube number	Homogenate solutions (mL) Spiked[b]	Unspiked[c]	Volume of TCA (mL)	µmol/L [KSCN]	Absorbance[d]
1	2.0	0.0	2.0	1000	0.446
2	1.2	0.8	2.0	600	0.250
3	0.8	1.2	2.0	400	0.175
4	0.2	1.8	2.0	100	0.040
5	0.0	2.0	2.0	--	0.000

a. Assay Protocol: this assay was carried out similar to that described in Table X.

b. Spiked by adding 10 µL of a solution containing 2.0×10^5 µmol/L KSCN to every 2.0 mL homogenate.

c. Added 10 µL distilled deionized water to every 2.0 mL homogenate.

d. The absorbances are the average of triplicate analyses.

precipitate all of the protein large volumes of zinc sulphate and either barium hydroxide or sodium hydroxide were required. Formaldehyde and chloral hydrate produce a gel from which the liquid containing the thiocyanate proved impossible to recover. Even high-speed centrifugation did not separate the liquid from the gel. These last two agents were expected to be useful because of their ability to trap cyanide thereby preventing further conversion to thiocyanate. Furthermore, formaldehyde prevents the formation of the iron-thiosulphate complex (75, 77). This combination would result in a blue colour interfering with iron-thiocyanate measurements. Unfortunately, addition of formaldehyde results in a gelation of the original solution.

It is probable that this gelation was due to the amount of protein in solution and the need to avoid diluting the sample. Determinations of the protein content of the homogenate were carried out using the Biuret reaction. An average of 10 g protein/100 mL homogenate was found after the initial centrifugation (prior to analysis). A slightly higher level of 10.7 g protein/100 mL homogenate was found prior to this centrifugation.

The assay for rhodanese was carried out using

potassium thiosulphate and potassium cyanide as substrates. Thiosulphate concentrations were five times those of cyanide. This follows the practice of Sörbo (77) and Schlesinger and Westley (92). The cyanide and thiosulphate levels in the reaction vessel were initially 0.01 and 0.05 M, respectively. These concentrations turned out to be the optimum substrate levels tested.

TCA was found to be the best precipitant, removing the protein and not interfering with the thiocyanate measurement. However the presence of cyanide and an acid required new safety measures. A cyanide trap consisting of a saturated sodium hydroxide solution and a cyanide indicator (chlorine, sulphanilic acid and pyridine in a sodium hydroxide - boric acid buffer) in sequential filter flasks were attached to a vacuum dessicator. A third filter flask was added and attached to a vacuum line. The centrifuge tubes containing the sample in the TCA were immediately placed in the dessicator. The dessicator-trap-indicator system was evacuated for 15 minutes. Any cyanide gas evolved was trapped in the base; the indicator remained clear and colourless showing that no cyanide escaped the trapping solution.

The centrifuge tubes were then sealed and spun at 15,000 rpm (39,000 x g) for 20 minutes. The supernatant was then poured off into a test tube. An aliquot of this solution was then tested for thiocyanate. Attempts were made to use the optimum conditions discussed in CHAPTER III PART I. However, this was not possible due to unknown matrix problems. A variety of reagent to sample volume ratios were tested, with 0.25 mL reagent to 1.00 mL sample being selected as most convenient and giving adequate absorbance. Two milliliters of hydrochloric acid per 100 mL of reagent was employed. Table XII shows some typical results. The high absorbances in such short time periods may be due to high levels of enzyme present.

TABLE XII

ASSAY FOR RHODANESE ACTIVITY[a]

Tube number	Time in seconds	Absorbance [c]	Thiocyanate µmol/L [b]
1	20	0.482	1032
2	40	0.935	2102
3	60	1.241	2785
4	90	1.350	3028
5	180	1.403	3146
6	300	1.519	3404

a. Assay Protocol: One mL of potassium thiosulphate and one mL of potassium cyanide were pipetted into the reaction vessel (final concentrations were 0.05 M and 0.01 M, respectively). An aliquot of the liver homogenate was added and another aliquot withdrawn at the specified times.

b. Calculated from standard curve data Table XI. The curve was extrapolated and checked at thiocyanate concentrations of 1500, 2000 and 3000 µmol/L. Linearity was maintained to this concentration.

c. The absorbances are the average of triplicate analyses.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The testing of a crude tissue homogenate for enzyme activity can be misleading due to the presence of inhibitors, or enhancers. However, the enzyme rhodanese appears to be amenable to this type of assay. High levels of enzyme activity were found and this allows for more accurate measurement of the reaction products, such as thiocyanate. An initial reaction rate, approximately 3000 μmol of thiocyanate, per minute was produced. In fact, dilution could be considered if it were not so full of problems itself.

Of all the precipitants or reaction stopping agents, TCA proved to be the most workable. A trap was employed to reduce the danger of poisoning by cyanide gas.

This system holds promise for accurately evaluating rhodanese levels in tissues of interest. Comparisons to other results were not made as the goals of this work had been completed.

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References 1, 3, 4 and 11 are papers related to thiocyanate assays.

Reference 2 deals with tungsten determinations as a complex with thiocyanate.

Reference 5 is related to the administration of cyanide and determination of thiocyanate.

References 6 and 7 are concerned with the hypotensive activity of thiocyanate and other anions.

References 8 and 9 are concerned with the ingestion of cyanide in cassava and its effects.

Reference 12 deals with thiocyanate and Red Sea brine.

References 13, 14 and 15 are related to cyanide poisoning.

References 16 and 17 are methods for studying enzyme activity.

References 18-26 concern the laetrile controversy.

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